# Ectendomycorrhizae in *Pinus resinosa* seedlings. I. Characteristics of mycorrhizae produced by a black imperfect fungus

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A black imperfect fungus isolated from 3-year-old nursery seedlings of *Pinus resinosa* was tested for its ability to form mycorrhizae on red pine in monoxenic culture. It was found to stimulate seedling growth. The fungal invasion passed through three successive stages of development during the 7 months after inoculation. At 3 months, intracellular hyphae occurred in all long- and short-root branches, with intercellular hyphae scattered only at isolated loci in the intercellular spaces. After 5 months the infection in the cortex changed abruptly from an intracellular infection to a completely intercellular Hartig net. Although the Hartig net was remarkably thin when first initiated, at its culmination it reached a massive thickness and sent penetrating hyphae into the cortical cells, resulting in the formation of distinctive ectendomycorrhizae. By the end of 7 months the secondary intracellular invasions were often found in the vicinity of the meristems in all root branches except the largest-diameter long-roots, and the fungus–root association had developed toward a predominant ectendomycorrhizal condition. The ectendomycorrhizal infection was distinctively different from the one produced by the so-called E-strains that have been commonly observed in coniferous nurseries.

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Les auteurs ont déterminé, sur des pins rouges monoxéniques, le potentiel mycorrhizateur d'un champignon imparfait à mycélium noir, isolé à partir de plants de *Pinus resinosa* produits en pépinière et âgés de 3 ans. Un effet stimulant a été observé sur la croissance. L'invasion par le champignon s'est faite en trois phases successives de développement au cours d'une période de 7 mois suivant l'inoculation. A l'âge de 3 mois, des hyphes intracellulaires se retrouvaient dans toutes les racines, longues et courtes, alors que des hyphes intercellulaires étaient dispersés à quelques endroits seulement dans les espaces intercellulaires. Après 5 mois, l'infection corticole s'est brusquement transformée, passant de l'infection intracellulaire à un réseau de Hartig complètement intercellulaire. Bien que le réseau de Hartig était particulièrement mince au début de sa formation il atteignit, au plus fort de son développement, une épaisseur considérable, émettant des hyphes de pénétration dans les cellules corticoles pour former des ectendomycorrhizes bien caractérisées. Vers la fin du 7ième mois, les invasions intracellulaires secondaires se retrouvaient souvent au voisinage des méristèmes dans toutes les ramifications racinaires, excepté dans les racines longues les plus grosses; l'association racine–champignon s'était alors développée vers un état ectendomycorrhizique prédominant. Les ectendomycorrhizes formées étaient nettement différentes de celles produites par les souches nommées "E-strains" qui ont été couramment observées dans les pépinières. [Traduit par le journal]

## Introduction

Investigators of mycorrhizae in coniferous nurseries have often encountered ectendomycorrhizal infections with intracellular infections of a characteristic type (Rayner 1934; Björkman 1942; Levisohn 1954; Mikola 1965; Wilcox 1968, 1971). The intracellular hyphae are large in diameter and heavily ramified throughout the cortex of both long- and short-roots. The fungal mantle is often relatively inconspicuous, and a Hartig net of thick hyphae surrounds the cortical cells in the small- and medium-diameter roots. In the larger roots the fungi may not form a Hartig net but remain scattered in the intercellular spaces or form only a rudimentary net.

Mikola (1965) isolated a so-called "E-strain" fungus from coniferous seedlings in Finnish nurseries and showed it to be responsible for the above described ectendomycorrhizae. Laiho (1965) performed experiments with Finnish E-strains and synthesized ectendomycorrhizae on various *Pinus* and *Larix* species. Since he found no other type of ectendomycorrhizae, he concluded that if others exist, they must be very rare, very local, or poorly defined.

Wilcox (1968) reported that ectendomycor-

rhizae with coarse hyphae as described by Mikola (1965) were common in nursery seedlings of *Pinus resinosa*. Subsequent studies of ectendomycorrhizae in red pine have detected other types not previously reported. The purpose of the present paper is to describe one of these produced by a fungus with small-diameter hyphae, designated as BDD-22.

## Materials and Methods

Fungus BDD-22 (Fig. 4) was isolated in March 1969 from 3-year-old seedlings of *Pinus resinosa* obtained from the forest nursery of the College of Environmental Science and Forestry at Syracuse, N.Y. The fungi were obtained from mycorrhizal short-roots after 15 min sterilization in 1% sodium hypochlorite and plating on an agar–gelatin medium that was a modification of Mikola's (1965) modified Hagem agar. The composition of this medium was as follows: $KH_2PO_4$, 0.5 g; $K_2HPO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $NH_4Cl$, 0.5 g; Fe citrate, 10 mg; $A_5$ supplemental micronutrients (Arnon 1940), 1 ml; glucose, 5.0 g; thymine, 50 $\mu$g; coconut milk, 50 ml; yeast extract, 100 mg; agar, 7.0 g; gelatin, 10.0 g; water, 1 liter; pH adjusted to 5.5. The fungal cultures were maintained on this medium at 20°C.

The procedure for monoxenic culture of mycorrhizae used in this investigation was a modification of that recommended by Marx and Zak (1965). Red pine seed was sterilized with 40% $H_2O_2$ for 20 min and germinated in test tubes on 1.5% cornmeal agar. After 4 weeks, uniform seedlings were selected and transferred to 2-liter flasks containing 750 ml of a sterilized vermiculite – peat moss substrate (99:1) moistened with an equal amount of the growing solution but without the gelatin and agar. Two months later the seedlings were inoculated with two 1- to 1½-cm discs from actively growing colonies of BDD-22. The flasks were incubated in a growth chamber under 1500 ft-c of combined incandescent and fluorescent illumination with a photoperiod of 16 h. The temperature was 24°C during the day and 18°C at night. Uninoculated control seedlings were grown under the same conditions.

The mycorrhizal synthesis experiments were performed twice. Inoculated and control seedlings (three or four of each) were harvested and photographed at about 3, 5, and 7 months after inoculation. Bits of substrate and portions of root were placed on Hagem agar plates to detect contamination and to reisolate the fungus. Portions of roots were removed and processed for slide preparation. The stains used were Pianese III-B (Wilcox and Marsh 1964) and Conant's quadruple stain (Triarch, Ripon, Wisconsin). The rest of the root system was cleared and stained in its entirety by the procedures of Bevege (1968). Cleared roots were examined by phase microscopy and selected portions were macerated by hand under the binocular microscope with specially prepared fine needles.

## Observations

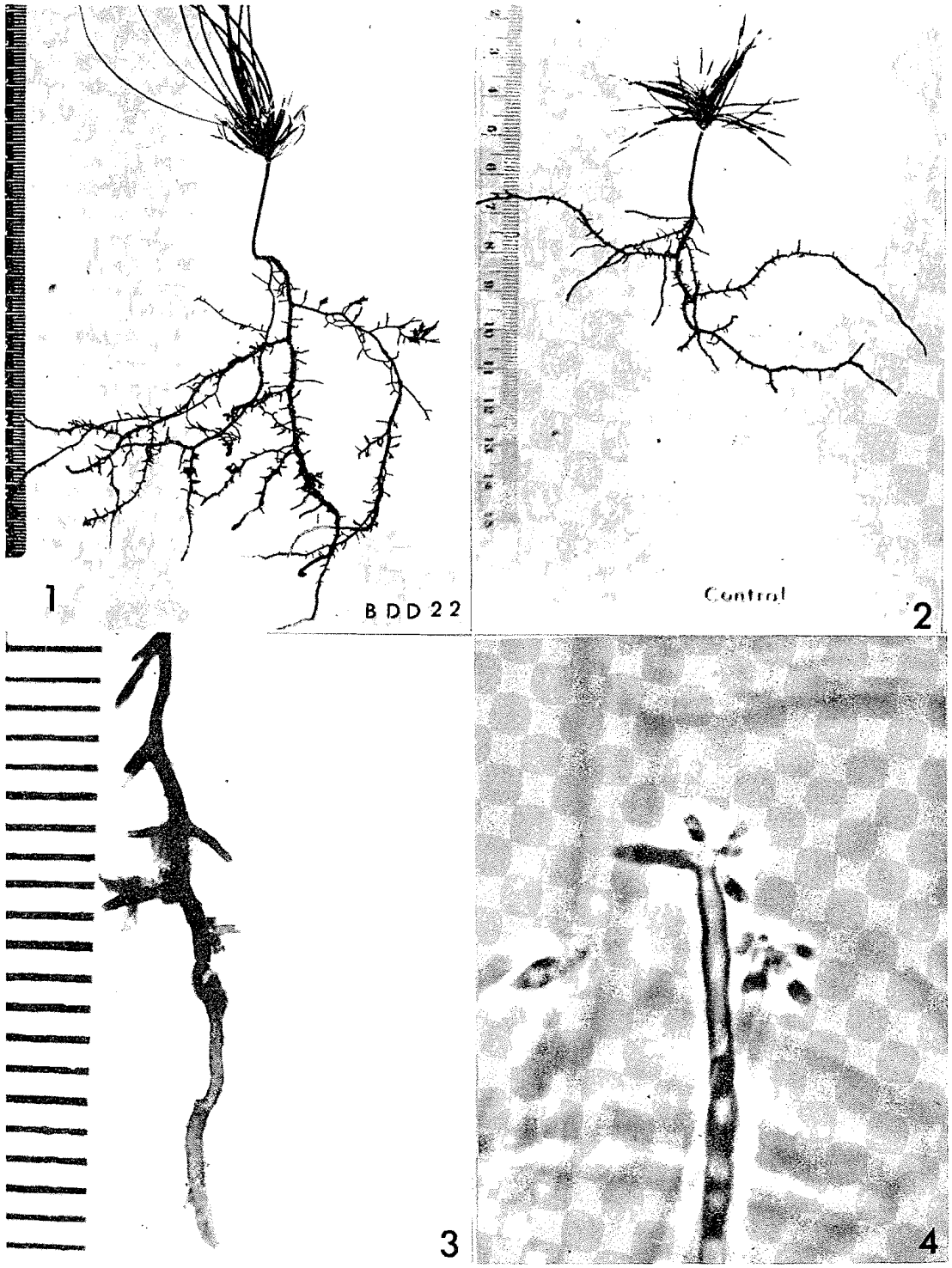
### *Gross Morphology of Red Pine Root Systems Colonized by BDD-22*

Seedlings invaded by BDD-22 possess larger root systems than control seedlings. This difference is already conspicuous at the first sampling period 3 months after inoculation (Figs. 1, 2). Both root systems are heterorhizic and display a prominent taproot and 5–10 first-order laterals, all with numerous short-root branches. The primary roots and first-order laterals of the seedlings with BDD-22 are longer than those of the controls. The first-order laterals near the base of the root system have produced second-order long-root branches in all the infected seedlings and in a few of the more vigorous control seedlings. These are also longer and more numerous in the infected seedlings than in any of the control seedlings.

Close observation of the infected seedlings shows a black fungus mantle on the surface of the long-roots. This mantle not only darkens the overall appearance of the root system but gives a dusky cast to the otherwise translucent unsuberized root apices (Fig. 3). The mantle is not uniformly developed over the surfaces but is aggregated here and there into small flocculent masses. In the uninfected seedlings the long-roots, in contrast, vary from light red-brown to dark brown with white translucent apices of varying lengths.

The size differences between infected and control seedlings become progressively greater in the sampling periods 5 and 7 months after inoculation. The short-root branches remain predominantly monopodial and are similar in appearance in both control and infected seedlings, although they usually are more numerous in the latter. The preponderance of these remain thin and unforked, but clusters of bifurcate short-roots can be found in seedlings 7 months after inoculation. However, these clusters are few, the branches are short and obscured by tufts of black hyphae, and the bifurcations could

FIG. 1. Five-month seedlings of red pine, 3 months after inoculation with fungus BDD-22. FIG. 2. Uninfected 5-month seedling of red pine. FIG. 3. Terminal portion of first-order lateral root of red pine, 3 months after inoculation with BDD-22. Scale in millimeters. FIG. 4. Conidiophore of BDD-22. × 1260.


1
BDD 22
Control
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be easily overlooked. As a consequence the root system might be dismissed as non-mycorrhizal by those investigators who depend upon short-root bifurcation as the sine qua non of mycorrhization.

*Growth of BDD-22 on Root Surfaces of Red Pine*

During the first 2–3 months after inoculation, the primary root as well as the first-order laterals shows a wispy hyphal mantle. Although most of the individual hyphal strands are more or less randomly disposed on the root surface, they occasionally parallel the axis of the root. These hyphae are conspicuous because of their straightness and their tendency to fuse into multicellular strands. These strands often lie over the common longitudinal wall between adjacent cortical cells. The individual hyphae of these strands have a diameter of about 3 microns ($\mu$) and have septae at intervals of 30–60 $\mu$. They are dark brown in color and may be either smooth or incrustate. The individual hyphae in the strands may separate as well as come together and, when following along the junction of two cells, may send out branches which penetrate between the cells. The cortical cells often appear to separate beneath a strand, indicating a possible hydrolytic action from the fungus. Where hyphal strands encounter a root hair, individual hyphae may separate, encircle the root hair, and rejoin on the opposite side (Fig. 5). Hyphal branches penetrate the base of the root hair and grow intracellularly toward the apex, frequently occluding it with hyphae. These hyphae are closely septate, the individual cells forming what appear to be chains of chlamydospores.

Hyphae sometimes encircle a root hair, forming a helix extending from its base to its tip (Fig. 6). The spiral may consist of an individual hypha with root-hair surface between the pitches. At other times several hyphae may form a spiral with hyphal threads appressed to occupy the entire surface of the root hair. The resultant pattern resembles that of a unicellular Hartig net on the surface of macerated cortical cells.

The features just described are best seen at a stage before the investing hyphae become too thick. Later the clumping of hyphae at intervals along the roots and the darkening of the suberizing root surface obscure the details of hyphal pattern. Tufts or clumps of hyphae are the culmination of the discontinuous distribution that occurs along the root surface. These may occur along the long-root near its junction with laterals, but this is not always the case. The fungi may be absent at the point of insertion of a lateral root and may be present on an isolated zone along the root.

In the collections at 5 and 7 months after inoculation, the clumping becomes progressively more conspicuous because of the continued growth of the amassing hyphae. In addition the strands of parallel hyphae on the surfaces of the larger roots continue to augment their size and appear as multistranded rhizomorphs at nearly uniform intervals around the circumference of the root. These parallel rhizomorphs are interconnected by anastomosing hyphae or groups of hyphae. The accumulation of hyphae results in a thick fungal mantle, which can be stripped off the root surface as a thick sheet of mycelium with its superposed parallel rhizomorphs. Additional changes are evident inside the mantle 7 months after inoculation and will be described with the anatomical features of the root.

*Root Anatomy of Red Pine Seedlings Inoculated with BDD-22*

*Three Months after Inoculation*

Intracellular hyphae occur in all long- and short-root branches of the seedlings 3 months after inoculation. These hyphae vary in a complex manner both within and between root branches. Many factors influence the colonizing behavior of these hyphae, but perhaps most important among these are the size of the root and the rate of its elongation.

Large roots, 1.0–1.4 mm in diameter, possess 8–11 layers of cortical cells along a radius between the endodermis and root surface. Cells in the outermost and innermost layers are conspicuously smaller than those in the bulk of the cortex (Fig. 7). The smallest roots, 0.2–0.4 mm in diameter, possess three to five layers of cortical cells with only the outermost conspicuously smaller than the rest (Fig. 10). Roots with intermediate diameters have proportionate numbers of cortical cells along a radius.

In the large roots, which are characteristic of the primary root and the larger first-order laterals, intracellular hyphae are thin, 1–2 $\mu$, and sparsely branched (Fig. 7). Intercellular hyphae can also be seen in transections at isolated loci. These remain discrete and do not form an inter-

cellular net (Fig. 7). Intracellular and intercellular hyphae are found throughout the cortex, with concentrations of both being greater among the smaller-diameter inner and outer cortical cells than in the center of the cortex (Fig. 7). Generally, the hyphae in the outer cortical layers resemble those on the root surface, and the former appear to have resulted from centripetal growth of the latter. At other times, hyphae can be found scattered throughout the cortex without appearing on the root surface. The presence of long strands of sparsely branched hyphae extending longitudinally (Fig. 14), both intercellularly and intracellularly, indicates that the fungus is moving within the root along its axis.

The initial penetration of the root apparently takes place early with the fungus appearing at about the same time in the inner and outer cortex. At first only a few hyphae are seen in a root transection. These are most frequently seen as circular cross sections of hyphae lying close to the walls of the cells, both inside the cell and in the intercellular spaces (Figs. 7, 8). In some of the cells, occasional hyphal strands can be seen traversing the cell with little or no branching (Fig. 13).

After the initial stages of penetration, there is a progressive augmentation of hyphae throughout the cortex and an increasing diversification in hyphal forms. The fine hyphae, which initially pass from cell to cell with a few sparse branches in the larger cells of the central cortex, may increase their branching to form dendritic structures with long thin branches. Other hyphae are closely septate and form chains of cells that are either sausage-shaped or bulbous in appearance. The smaller cortical cells, except those opposite the endodermal passage cells at the protoxylem positions (Fig. 9), fill with the circular shapes of hyphae cut in cross section. The hyphal-free cells opposite the protoxylem possess a unique, uniformly green-staining cytoplasm. In the most heavily infected roots an increase occurs in the amount of hyphae on the root surface and surrounding the cells in the outer two or three layers of the cortex. Gradually the outer cortical cells are separated from one another and appear to become entangled in an inwardly extending plexus of hyphae.

Many of the features of root colonization in large-diameter roots are also evident in medium- and small-diameter long-roots belonging to the second-order and the less vigorous first-order branches. However, some differences are apparent and these become more evident as the roots decrease in diameter and alter their structure. With loss of the large central cortical cells there is disappearance of the thin hyphae that traverse them. A decrease in intercellular spaces largely eliminates the scattered, discrete sites of intercellular hyphae and results in the predominance of intracellular infection (except for the beginning of Hartig net formation in distal root portions, as described later). With the shortening of cortical cells there is the great diminution or the complete absence of those uniform straight hyphae with their major directional component paralleling the root axis. Consequently, the characteristics of the intracellular hyphae in the inner and outer cortex become more similar as the root becomes smaller (Figs. 10, 11). The cortical cells are heavily invaded and often packed with hyphae possessing intercalary swellings, chlamydosporous bodies, and bizarre bulbous forms (Figs. 11, 12). These resemble the hyphae already described for the large-diameter long-roots but occupy a larger part of the cell volume.

Macerated cells substantiate many of the above details of fungal invasion; but, in addition, they often reveal more clearly the interrelationship of intracellular and intercellular hyphae of each cortical cell type. The pronounced longitudinal component of the hyphae in the cortex of large- and intermediate-diameter long-roots is evident in the cortical cell of Fig. 14, which is filled with numerous parallel hyphae extending the length of the cell and resembling faggots. Often, short hyphal connections occur between adjacent fungal filaments (Fig. 13). At times, the longitudinal hyphae appear to segment into chains of chlamydospores (Figs. 15, 16). Although these are mostly intercalary, similar chlamydospore-like structures may be borne terminally on short branches. The longitudinal hyphae may be continuous through a file of cells or curve along the end wall before penetrating it (Fig. 14). This can be seen clearly in individual macerated cortical cells.

The intercellular hyphae of the outer cortex are loosely attached to the cortical cells and are easily dislodged in macerated preparations. In the inner cortex, however, they may be more firmly attached to the cortical cells by means of

branches penetrating the cell wall. These intercellular hyphae are relatively sparse and in macerated cells are difficult to delimit from the intracellular hyphae because both are tightly appressed to the cell wall and are similar in appearance.

*Five Months after Inoculation*

Although the beginnings of a Hartig net occur at 3 months, in the distal portions of second-order laterals and in the third-order laterals arising from them, the best development can be seen after 5 months. The transition zone in the root is marked by the change in the cortex from an intracellular infection to a complete intercellular net and no intracellular hyphae. This change is evident in medial longisections of the terminal portions of the second-order laterals. With the stain used, the cytoplasm of the cells with intracellular hyphae stains olive-green or greenish-orange and the cell walls stain red. In the cells surrounded by a Hartig net, the cytoplasm stains bright green and the walls are also green. The Hartig net in the transition zone first develops through the middle lamella on the inner tangential wall of the cortical cells adjacent to the endodermis (Fig. 19). The Hartig net gradually surrounds these cells and then expands acropetally and centripetally to occupy the entire cortex (Fig. 18). Thus, the longisection view shows the proximal zone of intracellularly-infected cortical cells overarching a distally expanding wedge of cortical tissue with an ectomycorrhizal infection (Fig. 17).

In the transition zone the Hartig net arises first around those inner cortical cells opposite the passage cells of the endodermis. In an acropetal series of serial transections the Hartig net is seen to spread from these cells both circumferentially and centrifugally in the cortex. In the small- and medium-diameter roots, the Hartig net soon occupies the entire root cortex (Fig. 18). In the larger-diameter long-roots, the outer two or three layers of cortical cells may retain an intracellular infection, while the rest of the cortical cells are entirely ectomycorrhizal in appearance.

The first evidence of the formation of the Hartig net may be seen in longisections or in macerated cortical sections as a spotted or punctate pattern on the cell wall (Fig. 20). These spots frequently appear in a linear arrangement spiralling the cell or as festoons arching across the wall. Although there is no discernible hyphal wall at this early stage of Hartig net, the arrangement suggests a very fine hyphal filament with strings of closely placed nuclei. These spots may become progressively larger and ultimately each spot may be seen to be enclosed in a cell that has a pronounced polygonal form (Fig. 21). The cells are tightly appressed against one another and form a labyrinthine pattern on the cortical cell wall (Fig. 21). The overall pattern appears fine-textured and in the long cortical cells of the larger roots the long axis of the polygons appears to trace a spiral around the cell (Fig. 22). The pitch of this spiral frequently parallels the fibrillar angle of the cortical cell wall. At first the Hartig net is unicellular in thickness and is so fine-textured that it easily escapes notice. However, the hyphae of the Hartig net continue to proliferate between the cortical cells and ultimately form a thick, mycelial layer (Fig. 23).

The transition between the intracellularly infected portion of root and the more distal zone with a Hartig net can also be seen from the surface appearance of the root (Fig. 24). The former zone is marked by the presence of root hairs of varying surface density, while the latter zone is devoid of root hairs. Hyphae on the root surface over the ectomycorrhizal zone are also altered in appearance from those on the proximal zone of the root. A mantle is formed, at first unicellular in thickness, resembling the labyrinthine pattern in the Hartig net. The mantle gradually thickens, and the pattern becomes obscured. Thus, in most respects, the root undergoes changes to become a classical-type ectomycorrhiza. However, the metamorphosis does not confer an active stimulation to root bifurcation.

Many of the emerging third-order laterals possess a Hartig net and lack the preliminary intracellular infection, thereby becoming immediately ectomycorrhizal. Later the fungus may invade the cell from the Hartig net and spherical fungal bodies can be seen in the basal portion of the ectomycorrhizal zone, which represents the initiation of an ectendomycorrhizal condition.

*Seven Months after Inoculation*

Although the Hartig net is remarkably thin and delicate when first initiated, at its culmination it reaches a massive thickness and sends penetrating hyphae into the cortical cells (Fig. 25), resulting in the formation of distinctive

ectendomycorrhizae (Figs. 26, 27, 28). The ectendomycorrhizal condition first occurs in the basal portions of the smaller-diameter roots as previously indicated, but by the end of 7 months the fungus appears to have become more vigorous and the newest intracellular invasions are often seen in the vicinity of the meristems, except in the largest-diameter long-roots. Although these invasions develop from the Hartig net, they often occur immediately subjacent to the extending front of intercellular hyphae. The proliferation of the intracellular hyphae often appears to develop concomitantly with the thickening of the Hartig net (Figs. 29, 30). The penetrating hyphae produce branched structures and beaded chains of hyphae similar to those of the original intracellular infection (Fig. 26, 27, 28). The invading hyphae appear to be attracted to the vicinity of the cell nucleus and actively branch around it (Fig. 25). The entire cell is often filled with proliferating hyphae (Figs. 28, 30). The spherical or spore-like nature of these hyphal masses is much more pronounced than in the initial intracellular infection after inoculation.

Under the conditions of the experiment, the period from 5 to 7 months after inoculation is one of active growth, although many roots are finishing their growth at the end of this time. However, the ends of the principal first-order laterals appear succulent at 7 months and are obviously still growing. The more vigorous second-order laterals are also still growing and producing active third-order laterals, but the less vigorous second-order laterals, particularly those borne on the basal portion of the mother root, have formed metacutized dormancy layers. Any third-order laterals on these dormant branches are also inactive.

Examination of a vigorous first-order lateral, with all its branches intact, reveals that the fungus has invaded all parts of this system, except the rapidly growing apex of the main root. The fungus is found throughout the rest of the root system, including most of the apices of the slower growing second-order laterals. Intracellular hyphae are found throughout the axis of the first-order lateral itself, except for about the distal quarter, where the transition to the ectomycorrhizal-type infection may occur. The transition is often not complete at the particular root level, or it may not be continuous along the root axis, and the two types of infection may alternate with each other. In the larger first-order laterals the ectomycorrhizal infection commonly occupies the inner cortex with an intracellular infection in the outer two or three cortical layers. The secondary intracellular invasion from the Hartig net is often delayed in the larger first-order laterals.

The larger second-order laterals also exhibit intracellular infections in their basal portions and show the transition to an intercellular net in their intermediate portions. The Hartig net, once formed, extends acropetally into the apical region of the lateral and subsequently develops intracellular invasions. In the medium- and smaller-diameter second-order laterals an ectendomycorrhizal infection extends into the region of the meristem and a heavy mantle develops around the root apex (Fig. 32).

Those second-order laterals which show little elongation, and can presumably be classified as short-roots, can have several types of infections. Those near the base of the mother root may have intracellular infections, those in the distal portion may be ectomycorrhizal or ectendomycorrhizal (Fig. 31), and those in intermediate zones may have intracellular infections in their basal portions and intercellular infections in their distal portions.

Third-order laterals show a similar diversity. Again, basal laterals may have intracellular, and distal laterals either intercellular or a combined infection. In the ectendomycorrhizal infections the intracellular hyphae are the result of invasion from the Hartig net and not from the initial intracellular invasion. There is no obvious relationship between the infection of the emerging third-order laterals and their mother roots at the points of insertion. Roots with a well-developed Hartig net often arise at positions on mother roots where there is a strong intracellular infection or where no infection is discernible. Third-order laterals are more frequently short-root in character than are second-order laterals, but most of these are monopodial and difficult to assign to a given root class. However, a few are bifurcate and can be assigned short-root status without equivocation.

The above indicates that the fungus–root association is developing towards a predominantly ectendomycorrhizal condition and that the Hartig net arises more quickly and is more

stable in the smaller-diameter roots than in the larger first-order laterals. The initial intracellular infection is not, however, a temporary phase in root colonization but persists and undergoes developmental changes along with the progressive growth of the Hartig net and its penetrating hyphae. These changes in fungal colonization continue after root elongation has ceased.

Some root apices of seedlings 7 months after inoculation are dormant with pronounced metacutized layers. The patterns of intercellular and intracellular infection are similar to those of the more active root system reported above, but the following additional changes can be

FIGS. 5–8. Details of early invasion of large-diameter long-root of red pine, 3 months after inoculation: *e*, endodermis; *c*, cortex; *h*, hyphae; *ih*, intracellular hyphae; *r*, root hair. Fig. 5. Surface view of root looking down on a root hair. Hyphal strands on surface of root encircling and penetrating base of root hair. × 500. Fig. 6. Hyphae forming a helical spiral around a root hair. × 500. Fig. 7. Transection showing concentration of intracellular hyphae in inner and outer cortex. Intercellular hyphae remain discrete in the intercellular spaces. × 220. Fig. 8. Transection of cortical cells showing circular outlines of intracellular hyphae lying next to the inner cell wall and with occasional hyphae traversing the cell. × 1280.

FIGS. 9–12. Details of early invasion of medium-diameter long-root of red pine, 3 months after inoculation: *e*, endodermis; *ic*, inner cortex; *ih*, intracellular hyphae; *oc*, outer cortex. Fig. 9. Transection showing exclusion of intracellular hyphae from tanniferous inner cortical cells opposite passage cells of the endodermis. Remaining cells adjacent to endodermis heavily invaded by fungus. × 135. Fig. 10. Transection with heavy intracellular invasion in inner cortex. × 200. Fig. 11. Transection with bulbous intracellular structures. × 325. Fig. 12. Longisection with complete occlusion of cortical cell by intracellular hyphae. × 640.

FIGS. 13–16. Cortical cells from macerated roots of red pine, 3 months after inoculation. Fig. 13. Transverse and longitudinal components of hyphal system in elongated cortical cells of a long-root. × 315. Fig. 14. Intracellular hyphae extending the length of a cell and bending along the end wall. × 720. Fig. 15. Septate intracellular hyphae with prominent nuclei in cell from medium-diameter long-root. × 1320. Fig. 16. Beaded strand of intracellular hyphae with both intercalary and lateral chlamydospore-like structures. From a medium-diameter long-root. × 800.

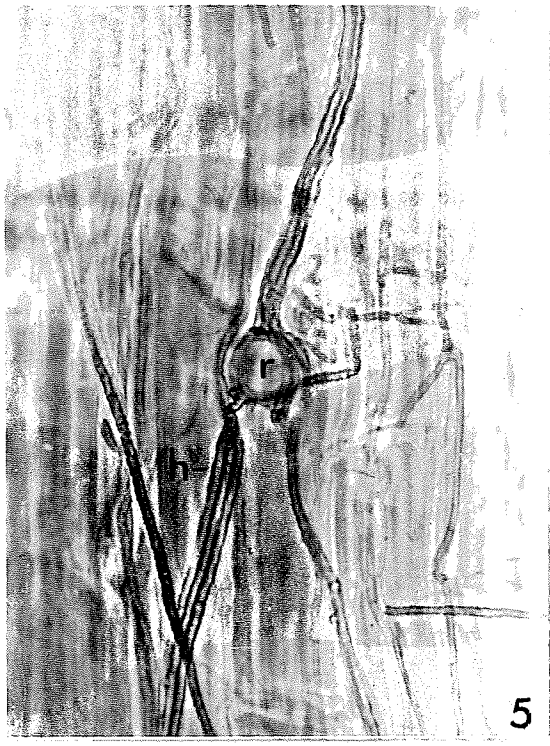
FIGS. 17–20. Early stages of Hartig net development, 5 months after inoculation: *e*, endodermis; *fm*, fungal mantle; *h*, Hartig net; *ih*, intracellular hyphae. Fig. 17. Longisection of small-diameter root with intracellular hyphae in outer cortex and beginning of Hartig net in the inner cortex. × 515. Fig. 18. Transection of small-diameter root with cortex fully occupied by a Hartig net and no intracellular infection. × 480. Fig. 19. Transection of small-diameter root showing intracellular infection in outer cortex and a well-developed Hartig net in the inner cortex. × 750. Fig. 20. Whole mount of cleared root, showing fine patterning of early Hartig net. × 490.

FIGS. 21–24. Stages of Hartig net development, 5 months after inoculation: *h*, Hartig net; *m*, mycorrhizal portion of root; *nm*, non-mycorrhizal portion of root; *rh*, root hair. Fig. 21. Fine labyrinthine pattern of Hartig net on a non-elongated cortical cell from vicinity of the apical meristem of a slow-growing long-root. × 1400. Fig. 22. Discontinuous Hartig net on elongated cortical cell from a long-root. Hartig net spirals around the cell at a pitch about that of fibrillar angle. × 640. Fig. 23. Continuous Hartig net on elongated cortical cell from a long-root. × 480. Fig. 24. Cleared, small-diameter root showing abrupt transition between the mycorrhizal region with a pronounced Hartig net and the non-mycorrhizal region without Hartig net but with root hairs. × 250.

FIGS. 25–28. Development of ectendomycorrhizal condition by intracellular invasion from the Hartig net, 5 months after inoculation: *cl*, chlamydospore-like intracellular structures; *h*, Hartig net; *ih*, intracellular hyphae; *n*, nucleus. Fig. 25. Longisection of long-root, showing manner of penetration and attraction of the fungus to the vicinity of the cell nucleus. × 560. Fig. 26. Longisection of long-root with chlamydospore-like intracellular structures characteristic of ectendomycorrhizal condition. × 300. Fig. 27. Longisection of small-diameter long-root with thin Hartig net and pronounced intracellular invasion. × 510. Fig. 28. Longisection of ectendomycorrhizal infection with thin Hartig net and varying sizes of spherical intracellular hyphal structures. × 700.

FIGS. 29–32. Advanced stages of ectendomycorrhizal development, 5 months after inoculation: *ai*, apical initials; *cl*, chlamydospore-like intracellular structures; *e*, endodermis; *fm*, fungus mantle; *h*, Hartig net; *m*, metacutized dormancy layer. Fig. 29. Macerated cells showing intracellular invasion from the Hartig net. × 815. Fig. 30. Macerated cells with Hartig net and intracellular invasion. Cell on left has more filamentous hyphae and the one on right a preponderance of chlamydospore-like spherical structures. × 1240. Fig. 31. Ectendomycorrhizal short-root with Hartig net developed in vicinity of meristem in advance of intracellular invasions. Root is dormant with well-developed mantle over root apex. × 475. Fig. 32. Ectendomycorrhizal long-root with heavy Hartig net and massive fungus mantle surrounding root apex. Root is inactive with developed dormancy layer. × 200.

FIGS. 33–36. Final stages of ectendomycorrhizal development, 5 months after inoculation: *c*, cortex; *fm*, fungus mantle; *h*, Hartig net, *sc*; senescing cortical cell; *sh*, fragmented senescing intracellular hyphae. Fig. 33. Transection of large-diameter long-root with thick, layered, gelatinous fungal mantle. Outer cortical cells are detached and trapped in the mantle. × 225. Fig. 34. Transection of large-diameter long-root with layered fungal mantle of lysed cortical and fungal tissues. × 1950. Fig. 35. Macerated cortical cell from the senescing cortex of a long-root. Cortical cell wall has lysed and intracellular hyphae are fragmented. × 685. Fig. 36. Macerated cells of long-root cortex showing appearance of senescing Hartig net. × 640.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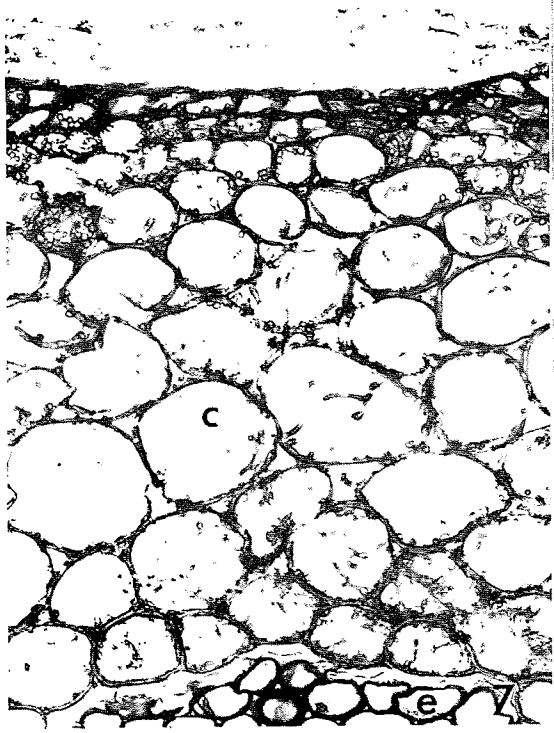
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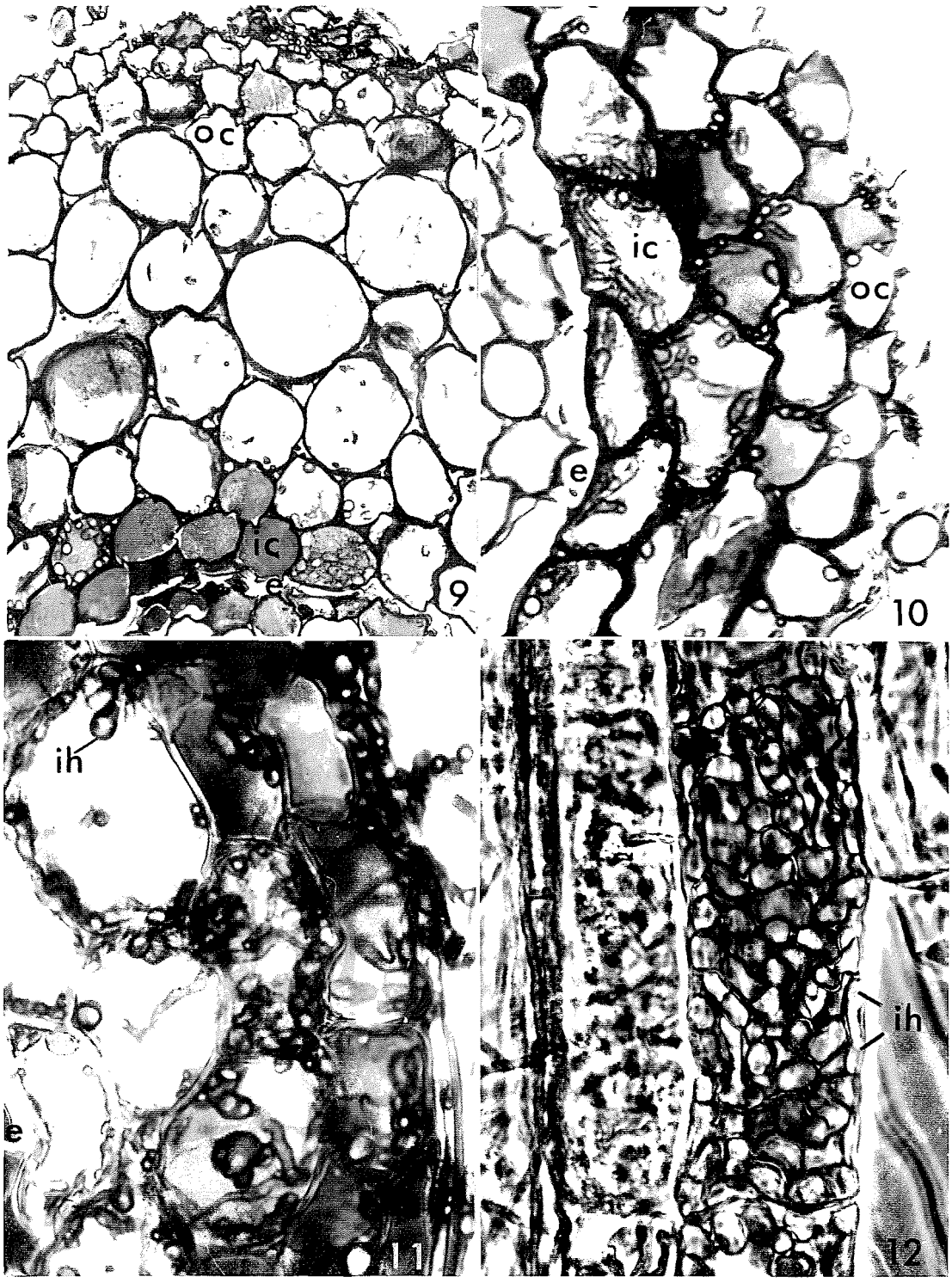
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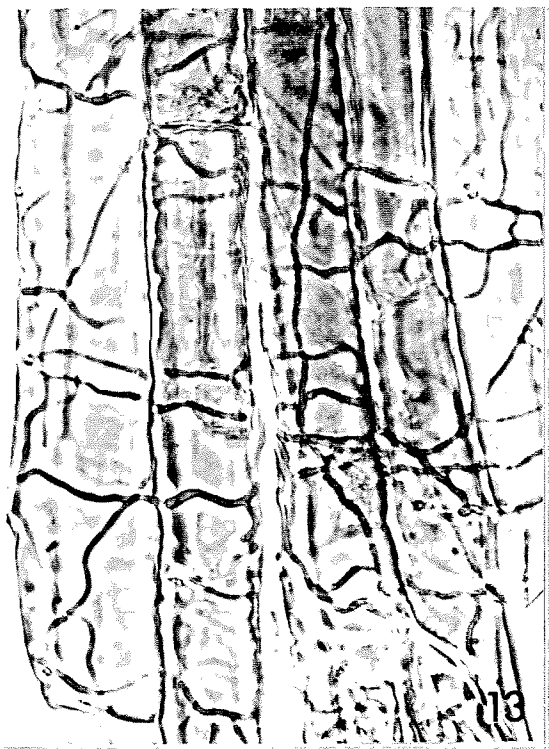

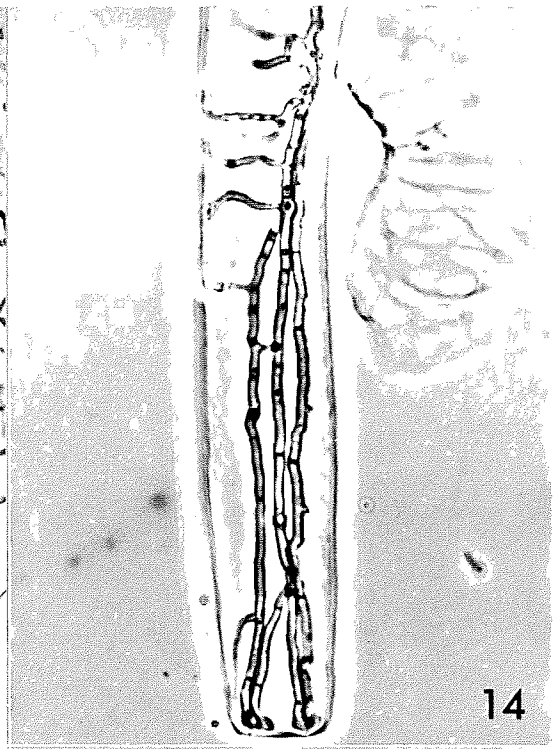
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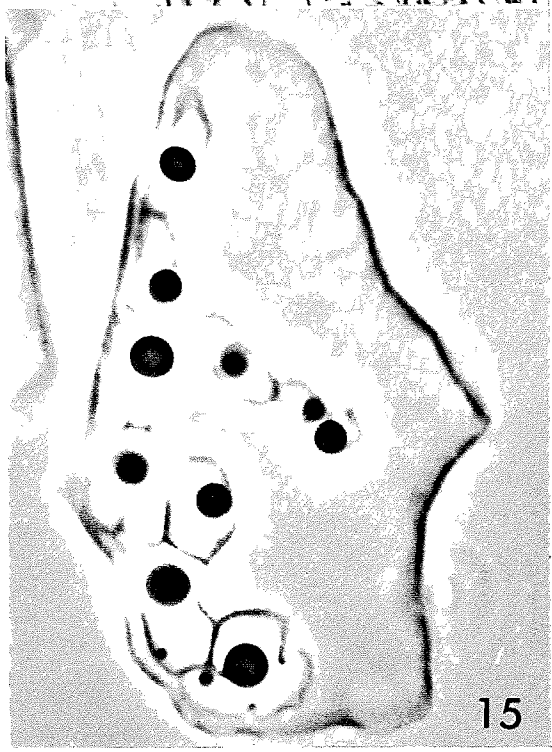
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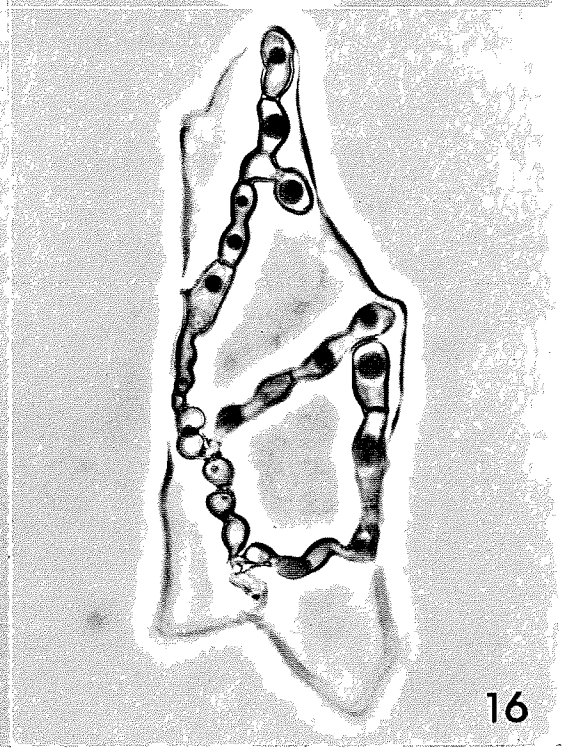
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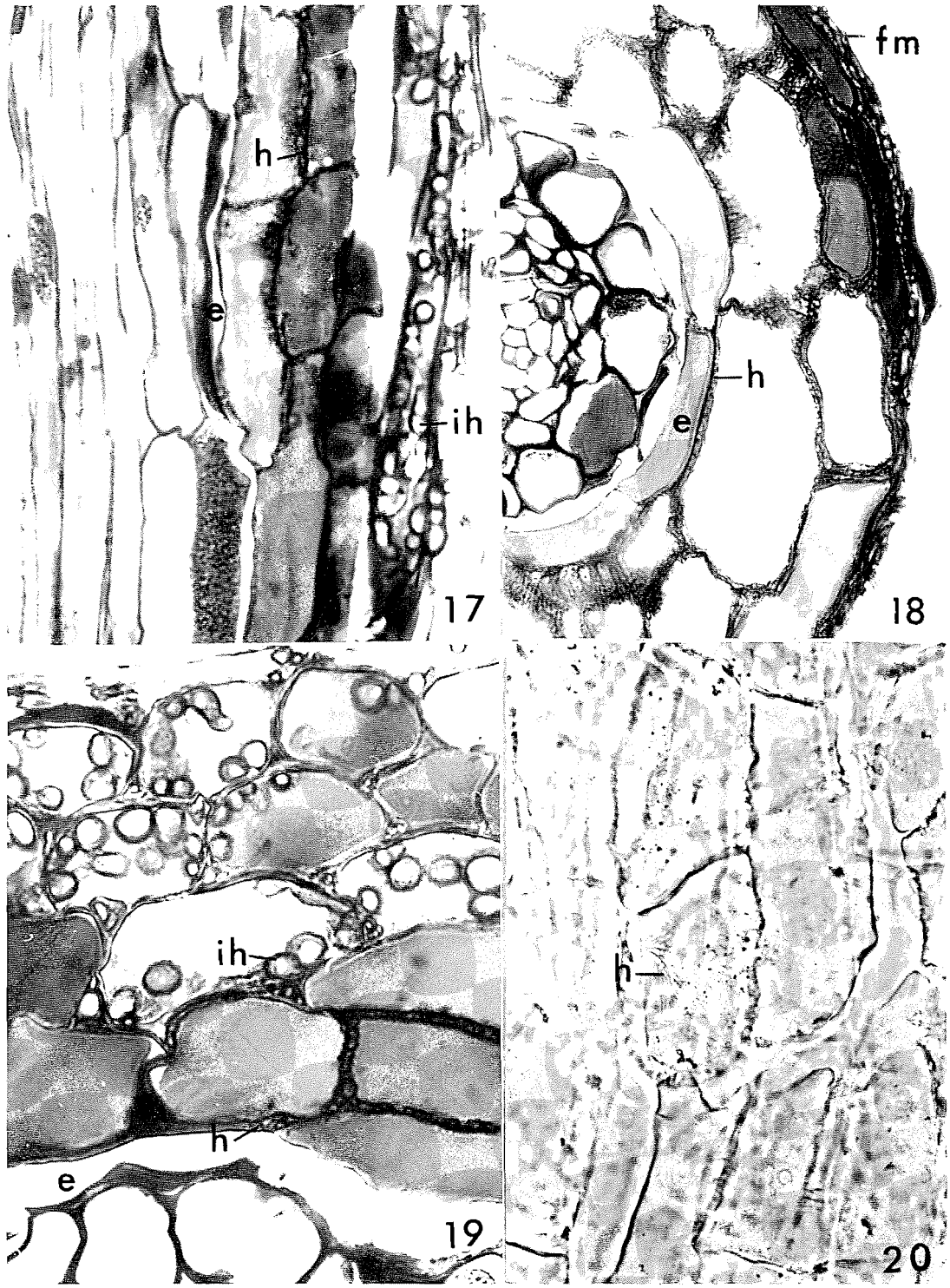
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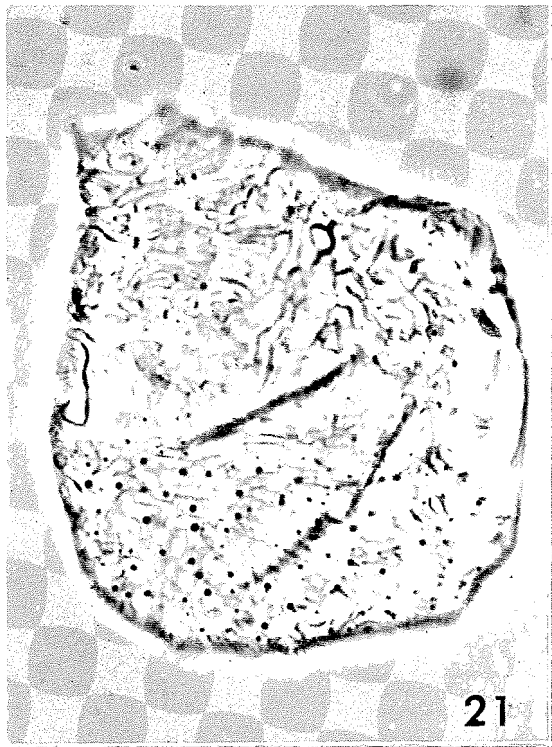
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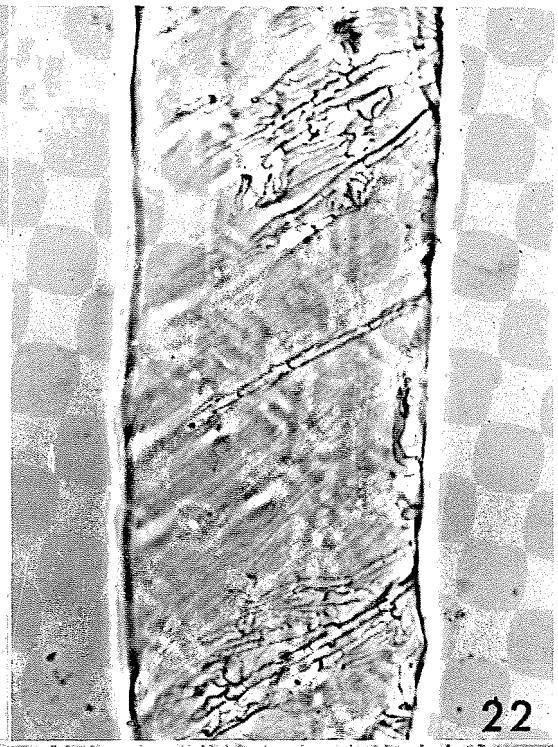
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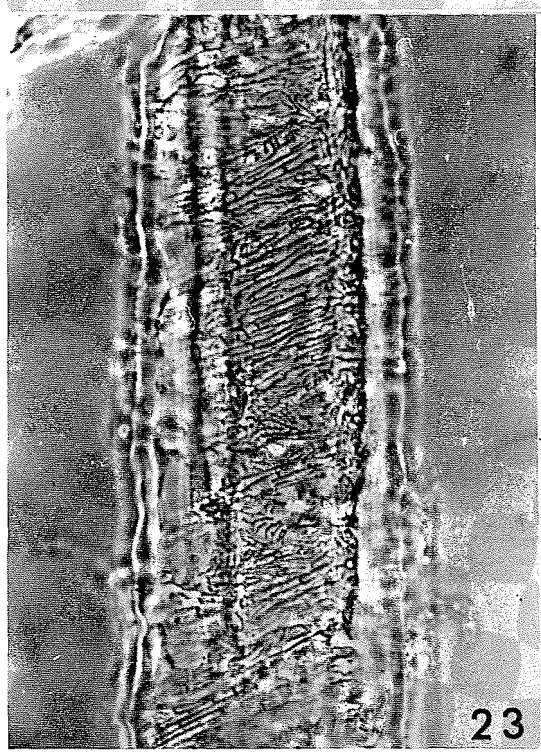
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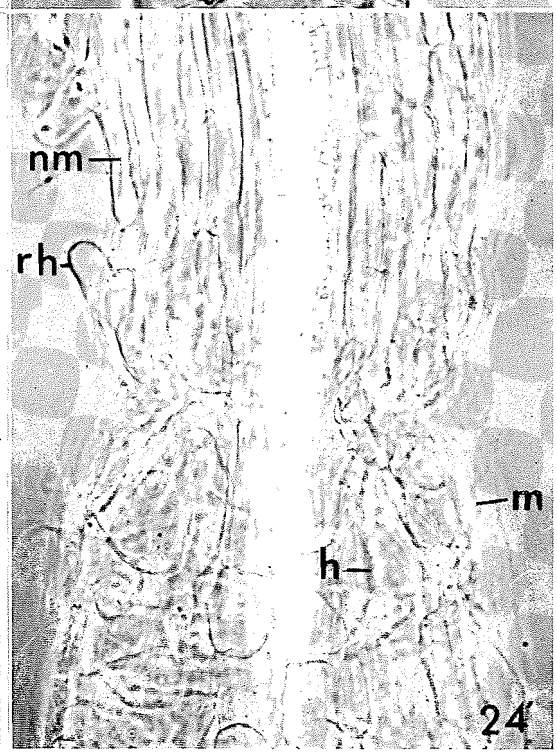
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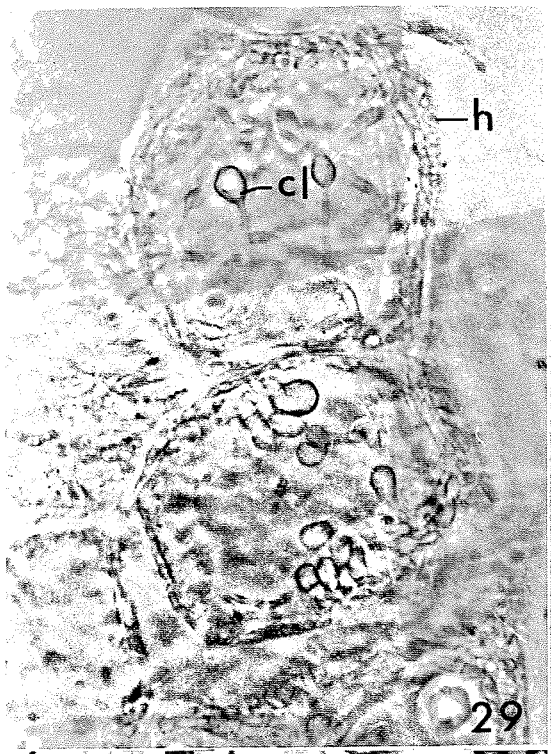


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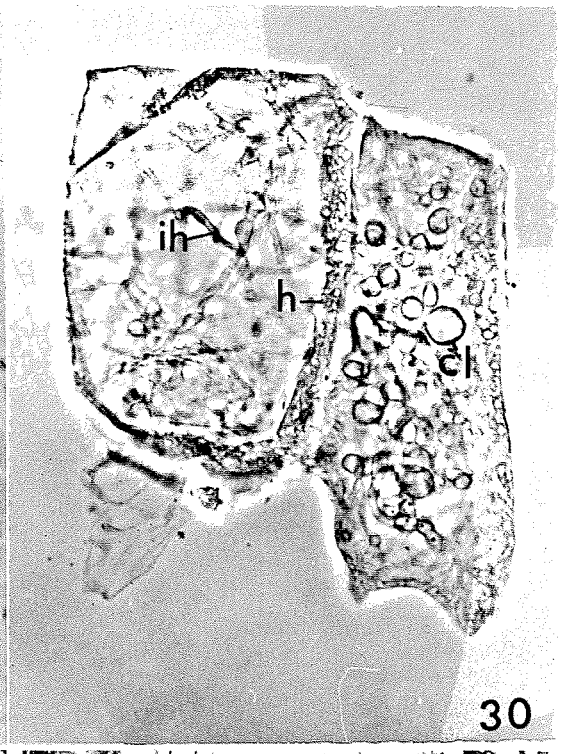


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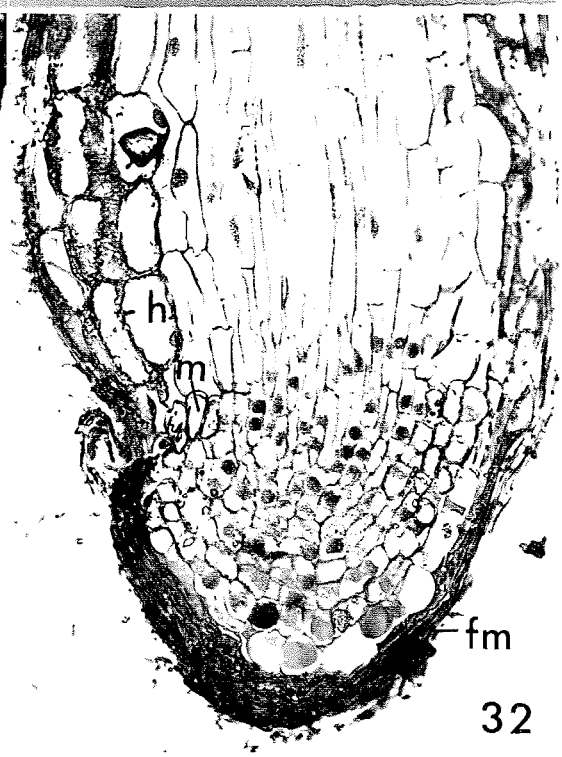


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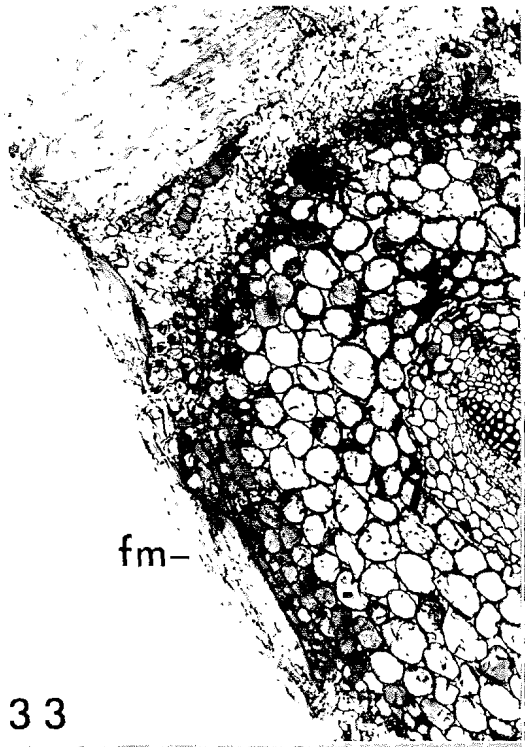


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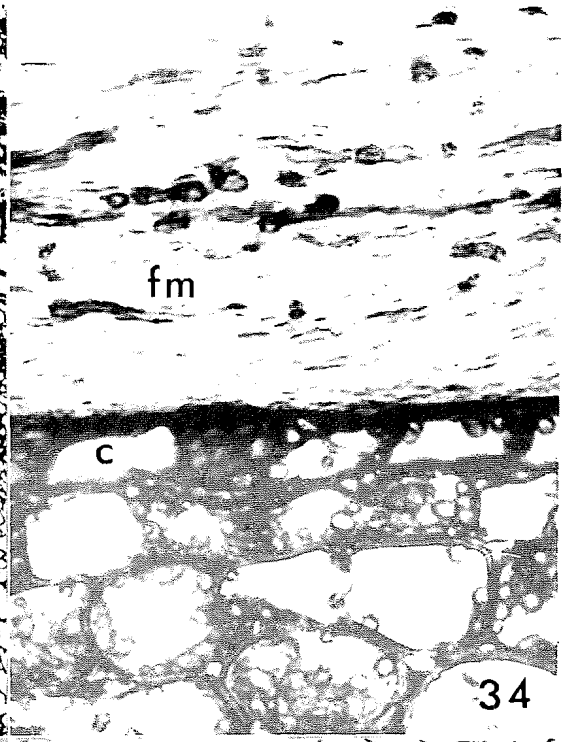


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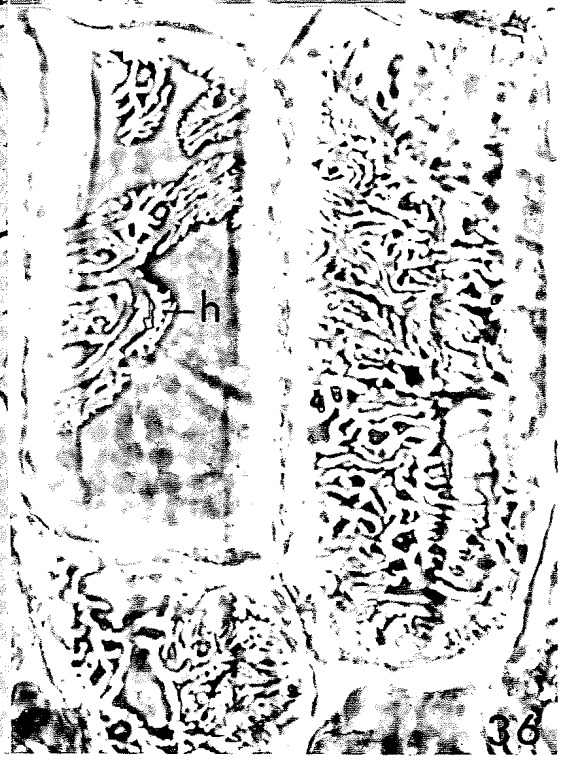


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noted. The initial intracellular infection has continued to increase its ramification in the cortices of the primary root and larger first-order laterals, and a thick mantle has developed on the root surfaces as already described. A transection through these roots shows that centrifugal to the mantle with its recognizable hyphae is a layer of substance which appears gelatinous but has a striated structure (Figs. 33, 34). Parallel to the striations are scattered short chains of spherical bodies that are spore-like in nature (Fig. 34). Examination of the transition region between the fungus mantle and the layered deposit shows the lysis of fungal hyphae and of cortical cells that have been enmeshed in the mantle (Fig. 35).

The first-formed Hartig net of these dormant roots is also undergoing senescence, which is indicated by the partial lysis of the cell walls and the deposit of unidentified chromogenic substances which cover the hyphal walls as well as the hyphal contents (Fig. 36).

## Discussion

The pronounced stimulation of seedling growth and development of red pine by the fungus BDD-22 is evidence of a beneficial association. The association ultimately leads to composite organs of root and fungus which have the features of a mycorrhiza, albeit a somewhat unusual one. Regardless of the particular mycorrhizal association formed, it is noteworthy that a black imperfect fungus, similar to the many commonly isolated from roots growing under natural conditions, is able to enter into symbiosis with red pine. This is remarkable because of the widespread beliefs that most such black fungi are parasites or secondary invaders (probably belonging to the form genus *Mycelium radicis atrovirens*) and that most mycorrhizal fungi of pine are Basidiomycetes. The results indicate the need for reexamination of these preconceptions. A wide range of fungi should be tested in mycorrhizal syntheses experiments; in particular, more attention should be paid to some of the ubiquitous black fungi which have been hitherto ignored.

Regarding the invasion of red pine by BDD-22, the question may be raised whether the characteristics of the initial infection, the ultimate ectendomycorrhizal infection, and the infrequency of bifurcation of the mycorrhiza are a result of cultural conditions or represent the spectrum of this association. It is not unprecedented for normally ectomycorrhizal fungi to produce endomycorrhizae in syntheses experiments. Melin (1923) performed such experiments with ectomycorrhizal fungi in aseptic sand cultures and found a shift to a more endotrophic mode of infection. He postulated three phases of mycorrhizal development: (1) the fungus penetrates and lives intracellularly, (2) the hyphae are largely digested and the fungus is displaced to the intercellular spaces, and (3) the fungus lives principally intercellularly but sends out occasional hyphae into the cells. He explained the dominance of the endotrophic and ectendotrophic infections in his syntheses as due to diminished fungus virulence. When the fungi are too weak they cannot invade, if somewhat more virulent they can invade the cortical cells but are too weak to grow between the cells, and when they are normally virulent they can develop between the cells after being displaced from them. He believed that the accumulation of toxins in the sand prevented the fungi from acquiring the necessary virulence to achieve the final ectomycorrhizal condition.

It is difficult to apply Melin's theory to the present results because no evidence was found of the digestion of the intracellular infection or its displacement to the intercellular spaces; rather, the Hartig net appears to originate in the intercellular spaces around cells that have never been invaded, such as the cortical cells opposite the passage cells in the endodermis. This sequence suggests that the final intracellular hyphae do not represent diminished virulence, but that the invasion of previously resistant cells from the Hartig net represents greater virulence of the fungus. However, this is a moot question. The observations of two periods of intracellular invasion may at least help explain the genesis of the controversy over whether the formation of a Hartig net follows or precedes intracellular invasion. Melin (1923) observed the former sequence and most other investigators the latter (Hartley 1969). In fungus BDD-22 it occurs in both ways.

It can be adduced from other experiments in our laboratory that the ectendomycorrhizal condition does not stem from culture conditions but represents the culminating condition of the BDD – red pine association. Under the same

cultural conditions, solely ectomycorrhizal associations are formed with *Amanita muscaria*, *Suillus subluteus*, and three strains of *Thelephora terrestris*, indicating that ectomycorrhizal species do not shift to the endotrophic mode of infection in the vermiculite–peat mixtures as in Melin's (1923) sand cultures (unpublished data); rather, the cultural conditions seem to enhance development of the various associations according to their propensities. Ectendomycorrhizae identical with those in the nursery (Wilcox 1968) are produced in cultures with red pine by isolates of the so-called E-strain fungus.

If we accept the fact that the BDD-22 – red pine association inheres to the ectendomycorrhizal condition, it then becomes untenable to accept the presumptions of Laiho (1965) that most of the ectendomycorrhizal associations are due to the E-strain of fungus isolated by Mikola (1965). The failure to observe the ectendomycorrhizal associations caused by BDD-22 in the nursery may be because their fine intercellular hyphae are less evident than the conspicuously bulbous intercellular hyphae of the E-strain fungus. On the other hand, the intracellular hyphae of the two fungi are not different enough in appearance to be readily distinguishable. In view of the difficulties of discriminating between ectendomycorrhizal fungi, we must admit the likelihood that still other fungi are involved in such associations.

The significance of the intracellular invasions by BDD-22 has still other implications. If the fungus is able to produce cellulases to effect the original penetrations, why does it lose this ability for a time and then regain it? If production of cellulases is related to the glucose content of the root, as suggested by Melin (1948, 1953) and Norkrans (1950), the question changes to why there should be such fluctuations in sugar level.

Harley (1969) has proposed that extreme forms of ectotrophs possess an obligate dependence on their hosts and that endotrophs have varying ability to utilize carbohydrates or varying dependence on accessory nutritional factors. Although this theory is plausible, it is difficult to apply to a fungus such as BDD-22, which forms the complete spectrum of mycorrhizal forms on the same host. It is difficult to see how this behavior can be related to sequential changes in nutrient supplies, nutritional needs, or abilities to obtain and utilize nutrients. Also, in view of the demonstrated benefits of BDD-22 at all stages, it is difficult to accept the ectendomycorrhizal relationships as an alteration toward parasitism and the detriment of the host, as proposed by some investigators (Rayner and Levisohn 1941; Robertson 1954; Levisohn 1963; Richard and Fortin 1970).

Finally, the present study corroborates the senior author's earlier comments on the artificiality of characters used to discriminate among short-roots, long-roots, and mycorrhizae (Wilcox 1967, 1968). The fungus BDD-22 ultimately produces a pronounced Hartig net and a mantle on red pine roots of small, medium, and sometimes large diameters, be they long-root or short-root in other anatomical characteristics. In the absence of active root bifurcation, it becomes almost impossible to tell whether a monopodial mycorrhiza is a mycorrhizal short-root, which will branch by bifurcation, if at all, or a mycorrhizal long-root, which will branch racemosely. By the same token, if the shorter long-root branches are recognized as being mycorrhizal, it becomes no longer tenable to refer to bifurcate short-root branches as epitomizing the ectomycorrhizal habit in *Pinus*, in distinction to the racemose mycorrhizal branching in, say, *Picea* or other members of the Pinaceae.

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